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Familial Hypercholesterolemia: Defective Binding of Lipoproteins to Cultured Fibroblasts Associated with Impaired Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity

(cholesterol synthesis/hyperlipidemia/atherosclerosis/enzyme regulation/receptors)

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ABSTRACT **Monolayers of cultured fibroblasts from normal human subjects bind ^{125}I-labeled low-density lipoproteins with high affinity and specificity. High affinity binding of similar magnitude was not observed in cells from five unrelated subjects with the homozygous form of familial hypercholesterolemia. In normal cells incubated at 37°, the binding sites were saturated at a low-density lipoprotein concentration of 20 μg/ml. A maximum of approximately 250,000 molecules could be bound to each cell. Whole serum and very-low-density lipoproteins displaced ^{125}I-labeled low-density lipoproteins from the binding sites, but high-density lipoproteins, the lipoprotein-deficient fraction of serum, and abetalipoproteinemic serum did not. This binding appears to be a required step in the process by which low-density lipoproteins normally suppress the synthesis of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-controlling enzyme in cholesterol biosynthesis. The demonstration of a defect in binding of low-density lipoproteins to cells from subjects with the homozygous form of familial hypercholesterolemia appears to explain the previously reported failure of lipoproteins to suppress the synthesis of this enzyme and hence may account for the overproduction of cholesterol that occurs in these cultured cells.**

In fibroblasts cultured from normal human subjects, the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) [mevalonate:NADP$^+$ oxidoreductase (CoA-acylating) EC 1.1.1.34], the rate-controlling enzyme in cholesterol biosynthesis, is regulated by the content of low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL) in the culture medium (1). In cells from subjects with the homozygous form of familial hypercholesterolemia (FH), an autosomal dominant disorder characterized clinically by hypercholesterolemia, xanthomas, and premature atherosclerosis (2), the synthesis of HMG CoA reductase molecules fails to be suppressed normally by LDL and VLDL (3, 4). Consequently, these mutant cells overproduce cholesterol (3). Our previous data suggested that this genetically-determined defect in enzyme regulation was due to an abnormality not in the structure of HMG CoA reductase itself but rather in a hitherto unidentified process involving the interaction of these mutant cells with extracellular lipoproteins (3, 4).

In the present studies we have investigated the interaction of [^{125}I]LDL with intact fibroblasts from normal subjects and individuals with homozygous FH. The data demonstrate that normal fibroblasts bind [^{125}I]LDL with high affinity and specificity and that this binding is deficient in the mutant cells.

Abbreviations: HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins; HDL, high-density lipoproteins; FH, familial hypercholesterolemia.

MATERIALS AND METHODS

Cells. Skin biopsies were obtained with informed consent, and fibroblast cultures were established in our laboratory as previously described (3–5). Clinical data on subjects from whom these cell lines were derived are given in Table 1 of this paper and Table 1 of ref. 5. In all experiments except as indicated, the normal cells came from subject no. 8 of ref. 5. The source of the cells from the homozygotes with FH used in the experiments in Figs. 2 and 3 is given in the appropriate legend. Cell lines were maintained in a humidified CO_2 incubator at 37° in 75-cm^2 flasks (Falcon) containing 10 ml of Eagle's minimum essential medium (Gibco, Cat. no. F-11), supplemented with penicillin (100 units/ml); streptomycin (100 μg/ml); 20 mM·tricine Cl (pH 7.4) (Sigma); 24 mM $NaHCO_3$; 1% (v/v) nonessential amino acids (Gibco); and 10% (v/v) fetal-calf serum (Flow Laboratories). For all experiments, cells from the stock flasks were dissociated with trypsin–EDTA solution (1) and were seeded (day 1) at a concentration of approximately 2.5×10^5 cells per dish into 60 × 15-mm dishes (Falcon) containing 3 ml of the above growth medium with 10% fetal-calf serum. On day 3 the medium was replaced with fresh growth medium containing 10% fetal-calf serum. On day 5 when the cells were confluent (average cell density, 9×10^5 cells per dish), the medium was removed and the cellular monolayer was washed with 2 ml of Dulbecco's phosphate-buffered saline (Gibco, Cat. no. 419), following which 2 ml of fresh medium containing 5% (v/v) lipoprotein-deficient human serum (final concentration of protein, 2.5 mg/ml) was added.

HMG CoA Reductase Assay. Cell extracts for measurement of rates of HMG CoA reductase activity were prepared as previously reported (1, 4). Aliquots (20–100 μg of protein) were incubated for 120 min at 37° in a final volume of 0.2 ml containing 0.1 M potassium phosphate (pH 7.5); 20 mM glucose-6-phosphate; 2.5 mM TPN; 0.7 unit glucose-6-phosphate dehydrogenase; 5 mM dithiothreitol; and 3×10^{-5} M DL [3-^{14}C]HMG CoA (5.26 Ci/mol). The [^{14}C]mevalonate formed was isolated by thin-layer chromatography and counted using an internal standard of [^{3}H]mevalonate to correct for incomplete recovery (5).

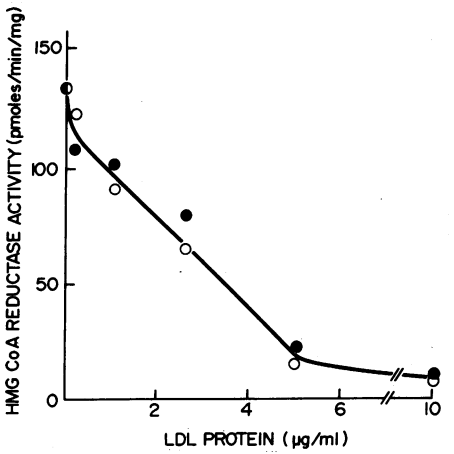

FIG. 1. Suppression of HMG CoA reductase activity of normal fibroblasts by [^{125}I]LDL and native LDL. On day 5 of cell growth, the medium was replaced with 3 ml of fresh medium containing 5 mg/ml of human lipoprotein-deficient serum protein. After 24 hr, 150 μl of buffer A containing varying amounts of either native LDL (○) or [^{126}I]LDL that had not been diluted with native LDL (●) was added to give the indicated protein concentrations. After 16 hr, extracts were prepared and assayed for HMG CoA reductase activity as described in *Materials and Methods*.

Lipoproteins. Human VLDL (d < 1.006 g/ml), LDL (d 1.019–1063 g/ml), HDL (d 1.063–1.215 g/ml), and lipoprotein-deficient serum (d > 1.215 g/ml) were isolated by sequential flotation in a Beckman preparative ultracentrifuge according to standard techniques (7) using solid KBr for density adjustment (8). Isolated fractions were dialyzed extensively against buffer containing 20 mM Tris·HCl (pH 7.4); 0.15 M NaCl; and 0.3 mM EDTA (buffer A) (4). Following dialysis, the lipoprotein-deficient fraction was defibrinated as previously reported (4). Blood from a patient with abetalipoproteinemia (4) and from a normal subject was collected in EDTA (1 mg/ml), and the resulting plasma samples were coagulated with thrombin (4) and dialyzed against buffer A. Protein concentrations were determined by a modification of the method of Lowry, *et al.* (9) using bovine-serum albumin as a standard.

[^{125}I]LDL. [^{125}I]LDL was prepared by Dr. David W. Bilheimer using a modification (10) of the iodine monochloride method of McFarlane (11). The final preparation, which was dialyzed into buffer A, contained less than 1 atom of iodine for each LDL molecule, and its final specific activity was 560 cpm/ng of protein. In this preparation, 99% of the radioactivity was precipitated by incubation with 15% trichloroacetic acid at 90°, 95% was precipitated by monospecific goat antiserum to human LDL (Hyland), and 2% was extractable into chloroform:methanol (12). When added to normal fibroblasts, this [^{125}I]LDL preparation was able to suppress HMG CoA reductase activity in a manner similar to that of native LDL (Fig. 1). For binding experiments, this preparation of [^{125}I]LDL was diluted with native LDL to give a final specific activity of 280 cpm/ng.

[^{125}I]LDL Binding to Intact Fibroblasts. Cells were grown in dishes as described above, and on day 6 after 24-hr growth in medium containing lipoprotein-deficient serum, the medium was removed from each dish and replaced with 2 ml of solution consisting of Eagle's minimum essential medium; 20 mM tricine·HCl, pH 7.4; and 2.5 mg/ml of lipoprotein-deficient serum (buffer B) and the indicated amounts of [^{125}I]LDL and native LDL, which were added in a volume of buffer A varying from 2 to 150 μl. After incubation for the indicated time either at 4° or 37°, the medium was removed and all subsequent operations were carried out at 4°. Each cell monolayer was washed three times in rapid succession with 3 ml of buffer containing 50 mM Tris·HCl (pH 7.4); 0.15 M NaCl; and 2 mg/ml of bovine-serum albumin (buffer C), after which a further 3 ml of buffer C was added and the monolayer was incubated for 2 min. This latter step was repeated once, each monolayer was washed finally with 3 ml of buffer containing 50 mM Tris·HCl (pH 7.4) and 0.15 M NaCl, and the cells were removed from the dish by dissolution in 1 ml of 0.1 N NaOH. Aliquots (500 μl and 50 μl, respectively) were removed from each dish for scintillation counting in a gamma counter and for measurement of protein concentration (9). In all figures, each point represents the value obtained from a single dish and is expressed as the ng of [^{125}I]LDL protein bound per mg of total cell protein. Each dish contained 350–500 μg of total cell protein. Duplicate determinations of [^{125}I]LDL binding varied less than 5% of the mean values.

RESULTS

When monolayers of normal fibroblasts were incubated with low concentrations of [^{125}I]LDL, a time and temperature-dependent binding of radioactivity to the cells was observed (Fig. 2, *left*). The amount of [^{125}I]LDL bound to the cells was reduced by the addition of a 50-fold excess of native LDL, suggesting that the [^{125}I]LDL and native LDL were competing for a limited number of specific binding sites. Specific binding (defined as the difference between the radioactivity bound in the absence and presence of excess native LDL) appeared to reach a plateau at approximately 3 hr. Both the initial rate and the extent of specific binding were greater at 37° than at 4° in the normal cells (Fig. 2).

In contrast to normal cells, fibroblasts from a homozygote with FH had virtually no detectable specific binding of [^{125}I]-LDL at either 4° or 37° at the LDL concentration used (Fig. 2, *right*). Nonspecific binding (defined as the radioactivity bound in the presence of an excess of native LDL) was similar in the mutant and normal cells (Fig. 2).

When the binding reaction was allowed to reach equilibrium at concentrations of LDL between 0 and 90 μg/ml, specific binding of [^{125}I]LDL to normal cells showed saturation kinetics (Fig. 3*A*, *left*). At 37°, saturation occurred at about 20 μg/ml of LDL, and half-maximal binding was observed in the range of 10 μg/ml. By contrast, the nonspecific binding of [^{125}I]LDL was not saturable and increased linearly with increasing amounts of LDL. At concentrations of LDL lower than 15 μg/ml, the ratio of specific to nonspecific binding was about 10:1. A Scatchard plot (16) of these data suggested the presence of a specific binding site of high affinity (Fig. 3*B*). The apparent dissociation constant, K_d, calculated from this plot, was 15 μg/ml (2.5×10^{-8} M, assuming a molecular weight for LDL of 3×10^6, of which the protein component comprises 20%, ref. 13), and the maximum amount of LDL bound at specific sites was 1.0 μg of LDL protein per mg of total cell protein. It was calculated that at 37° each cell is capable of binding a maximum of 250,000 molecules of LDL at this high-affinity binding site. When similar experiments were carried out at 4°, saturation kinetics were also observed. However,

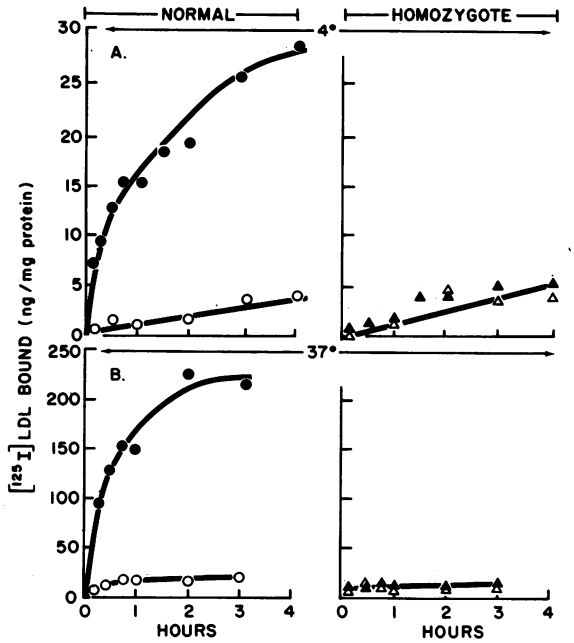

FIG. 2. Time course of [125I]LDL binding to intact cells from a normal subject (*circles*) and a homozygote with FH (*triangles*) at 4° and 37°. On day 6 of cell growth, the medium was removed and the cell monolayers were incubated for the indicated time at 4° (*A*) or 37° (*B*) in 2 ml of buffer B containing 5 μg/ml of [125I]LDL in the presence (○, △) and absence (●, ▲) of 250 μg/ml of native LDL. The cell monolayers were then washed and harvested as described in *Materials and Methods*. The mutant cells were those of subject no. 8 in Table 1.

while the apparent affinity was somewhat higher (K_d = 4.5 × 10^{-9} M), the maximum number of LDL molecules bound at 4° (7,500 molecules per cell) was only 1/30th that observed at 37° (data not shown). When fibroblasts that had been incubated at either 4° or 37° with [125I]LDL were disrupted in a Polytron homogenizer, 85% of the bound radioactivity was sedimented by centrifugation for 15 min at 10,000 × *g*, indicating that the [125I]LDL was bound to cell membrane fractions.

In cells from a homozygote with FH, virtually no specific binding could be detected over a wide range of LDL concentrations (Fig. 3*A*, *right*). However, nonspecific binding of LDL to these cells was similar to that of the normal. A Scatchard plot of these data confirmed the absence in the mutant cells of a high-affinity binding site for LDL (Fig. 3*B*).

The cells used in Figs. 2 and 3 were from two unrelated homozygotes (subjects no. 8 and 9, Table 1). A comparison of specific and nonspecific [125I]LDL binding in cells from these and three other unrelated homozygotes with that of seven normal cell lines is shown in Table 1. When tested at one nonsaturating level of LDL (5 μg/ml), mean specific binding in the normal cells at 4° was 4-fold greater than in the mutant cells, and at 37° there was a 9-fold difference. Although specific binding was deficient in the cells of all homozygotes tested, a 5- to 10-fold variation was observed in these low levels, among the different mutant cell lines. This was in contrast to the relative lack of variation among the normal cell lines.

The demonstration of high-affinity binding for LDL in normal cells and its apparent deficiency in cells from five unrelated homozygotes with FH, coupled with the previous observations that LDL suppresses HMG CoA reductase activity in normal cells but not in these mutant cells (3, 4), suggested that specific binding of LDL is a necessary step in this regulatory process. This hypothesis was supported by the experiments in Fig. 4, which show that when normal cells were incubated with LDL at a wide range of concentrations, the relative amount of binding was closely correlated with the relative degree of suppression of HMG CoA reductase activity.

Evidence that the observed 125I-binding to normal cells is specific for LDL was obtained in experiments shown in Figs. 5 and 6. When the [125I]LDL preparation was preincubated with varying amounts of an antibody to LDL, increasing amounts of the radioactivity were precipitated (Fig. 5). This resulted in a proportional decrease in the amount of radioactivity that could be bound specifically to the normal cells (Fig. 5). The specificity of LDL binding was further shown by the observations that whole serum, LDL, and VLDL competed with [125I]LDL for binding, but that HDL and the lipoprotein-deficient fraction of serum ($d > 1.215$ g/ml) showed much less ability to displace LDL (Fig. 6*A*). Since whole serum, LDL, and VLDL, but not HDL, contain apolipoprotein B (14), these data suggest that this apolipoprotein is important in the binding of LDL to fibroblasts. Consistent with this hypothesis is the observation that serum

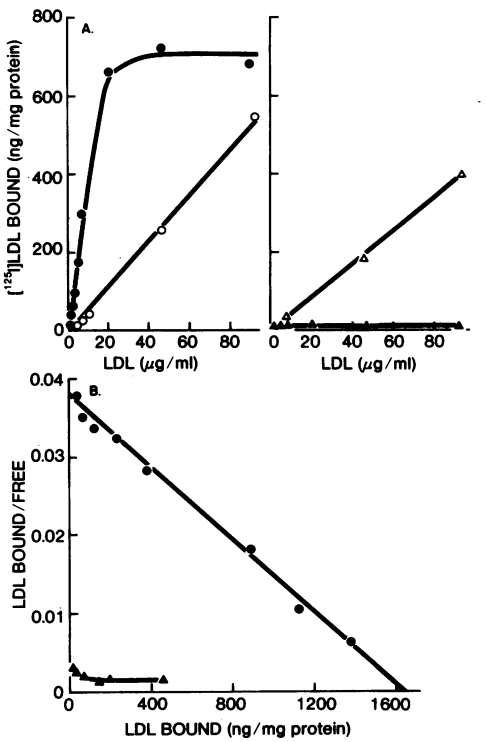

FIG. 3. [125I]LDL binding to intact cells from a normal subject and a homozygote with FH. On day 6 of cell growth, the medium was removed and the cell monolayers were incubated for 3 hr at 37° in 2 ml of buffer B containing the indicated concentration of [125I]LDL in the presence (○, △) and the absence of an additional 550 μg/ml of native LDL. The cell monolayers were washed and harvested as described in *Materials and Methods*. In *A*, the amount of specifically bound [125I]LDL (●, ▲) was determined by subtracting the radioactivity bound in the presence of native LDL from the radioactivity bound in the absence of native LDL. In *B*, [125I]LDL binding in normal (●) and homozygous (▲) cells is plotted by the method of Scatchard (16). "Bound/free" represents the total [125I]LDL bound (ng/mg of cell protein) divided by the total amount of LDL present in the medium (ng/2 ml). The mutant cells were those of subject no. 9 in Table 1.

TABLE 1. *[^{125}I]LDL binding to cells from normal subjects and homozygotes with familial hypercholesterolemia*

Subject	Sex	Age (years)	[^{125}I]LDL bound (ng/mg of protein) 4° Total	4° Nonspecific	4° Specific	37° Total	37° Nonspecific	37° Specific
Normals								
1 (8)*	M	Newborn	26.2	5.4	20.8	237	13.3	224
2 (1)	M	6	25.1	3.7	21.4	250	16.7	233
3 (6)	M	11	23.3	4.6	18.7			
4	F	31	29.3	4.2	25.1			
5	M	34				194	41.0	153
6	F	24				235	46.0	189
7	F	24				201	36.1	165
		Mean	26.0	4.5	21.5	223	30.6	193
Homozygotes								
8 (23)*	F	12	8.1	6.8	1.3	15.2	9.6	5.6
9 (24)	F	6	13.6	8.4	5.2	25.1	15.1	10.0
10 (25)	M	10	17.9	9.3	8.6	69.0	23.7	45.3
11 (26)	F	23	19.3	12.7	6.6	56.1	36.7	19.4
12	F	6	11.4	6.3	5.1	44.5	16.3	28.2
		Mean	14.1	8.7	5.4	42.0	20.3	21.7

On day 6 of cell growth, the medium was replaced with 2 ml of buffer B containing 5 μg/ml of [^{125}I]LDL either in the absence or presence of 500 μg/ml of native LDL. After incubation for 3 hr at either 4° or 37° the cell monolayers were washed and harvested as described in *Materials and Methods*. Specific binding was calculated by subtracting the [^{125}I]LDL bound in the presence of native LDL (nonspecific) from the [^{125}I]LDL bound in the absence of native LDL (total). Each value represents the mean of duplicate determinations.

* The number in parentheses refers to the number of each subject in Table 1 of ref. 5, where additional clinical data are given.

from a patient with abetalipoproteinemia, which is devoid of apolipoprotein B (15), was considerably less effective than normal serum in displacing [^{125}I]LDL from its binding sites (Fig. 6*B*).

The ^{125}I radioactivity bound to the normal cells at either 4° or 37° remained associated with protein as determined by its complete precipitation by 15% trichloroacetic acid at 90°. Only 2% of the bound radioactivity could be extracted by chloroform:methanol (12). When cells that had previously been incubated with [^{125}I]LDL were transferred to medium containing unlabeled LDL and incubated at 37°, the radioactivity was released from the cells by a first-order process (Fig. 7). This release of radioactivity was temperature-dependent (Fig. 7) and was associated with degradation of the [^{125}I]LDL molecule as determined by the fact that the majority of the released radioactivity was converted to a dialyzable form and less than 25% could be precipitated by either trichloroacetic acid or anti-LDL. The release of radioactivity from the cells at 4° and 37° was unaffected by the presence of levels of native LDL up to 500 μg/ml and occurred equally in the absence or presence of lipoprotein-deficient serum.

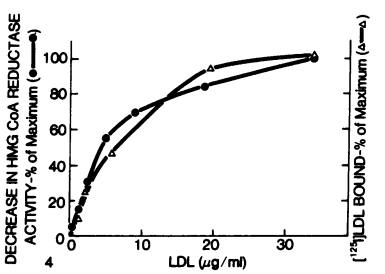

FIG. 4. Comparison of [^{125}I]LDL binding (△) and [^{125}I]LDL-mediated suppression of HMG CoA reductase activity (●) in intact normal cells. Cells were grown for 6 days in dishes as described in *Materials and Methods*. For the binding studies, monolayers were incubated for 3 hr at 37° in 2 ml of buffer B containing the indicated concentration of [^{125}I]LDL in the presence and absence of 250 μg/ml of native LDL. Specific [^{125}I]LDL binding at 35 μg/ml was 700 ng/mg of protein and this value was taken to represent 100% of maximum binding. For measurement of HMG CoA reductase activity, cell monolayers were incubated for 6 hr at 37° in 2 ml of fresh medium containing 5 mg/ml of human lipoprotein-deficient serum and the indicated concentration of [^{125}I]LDL. HMG CoA reductase activity in the absence of LDL was 82 pmols/min per mg of protein and was suppressed to 3.7 pmols/min per mg of protein in the presence of 35 μg/ml of LDL. The latter value was taken to represent 100% of the maximum decrease in enzyme activity.

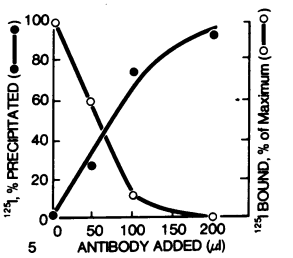

FIG. 5. Reduction of [^{125}I]LDL binding to normal cells by preincubation of [^{125}I]LDL with anti-LDL antibody. [^{125}I]LDL (50 μg) was incubated with each of the indicated amounts of a monospecific goat antibody to human LDL (Hyland) in a final volume of 200 μl for 2 hr at 37° and then for 12 hr at 4°. The immunoprecipitates were removed by centrifugation and aliquots of the supernatant from each incubation were taken for determination of radioactivity remaining after antibody precipitation. Specific [^{125}I]LDL binding to cells was determined by incubation of 25-μl aliquots of each supernatant in 2 ml of buffer B with cell monolayers for 3 hr at 37° in the presence and absence of 250 μg/ml of native LDL as described in *Materials and Methods*. The supernatant (25 μl) from the tube containing no anti-LDL (0% precipitated) contained 1.4×10^6 cpm, and of these cpm, 3.1×10^4 cpm were specifically bound by the cells (100% of maximum).

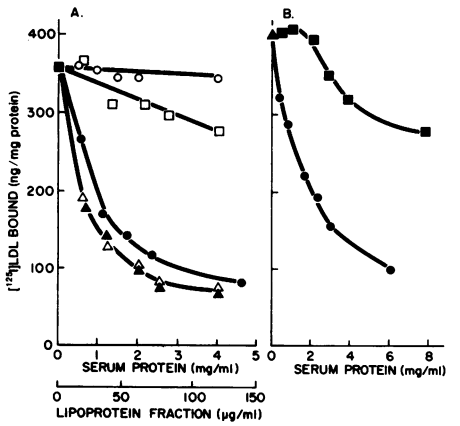

FIG. 6. Effect of serum and lipoprotein fractions on [^{125}I]LDL binding to normal fibroblasts. On day 6 of cell growth, the medium was removed and the cell monolayers were incubated for 3 hr at 37° in 2 ml of fresh medium containing 20 mM tricine·HCl (pH 7.4), 10 μg/ml of [^{125}I]LDL, and the indicated concentration of the following. *A:* ●, whole normal serum; ○, lipoprotein-deficient serum; □, HDL; △, LDL; ▲, VLDL. *B:* ●, whole normal serum; ■, whole abetalipoproteinemic serum. In all experiments, except those in which the concentration of lipoprotein-deficient serum was varied (○), the incubation medium contained 2.5 mg/ml of lipoprotein-deficient serum.

DISCUSSION

The present studies demonstrate that cultured fibroblasts from normal subjects take up [^{125}I]LDL by a temperature-dependent process that is highly specific, reaches an equilibrium with time, and is saturable at low levels of LDL. That this process represents binding to the cell membrane is suggested by the observation that the radioactivity that is taken up remains associated with the membrane fraction of the cell. We recognize, however, that the present data cannot exclude the possibility that part of the material regarded as "bound" has in fact been specifically transported into the cell. For simplicity, we have referred to the process under study as binding. That this binding is directly related to the LDL-mediated suppression of HMG CoA reductase activity is indicated by three observations: (*i*) the degree of suppression of HMG CoA reductase activity is proportional to the amount of LDL bound; (*ii*) VLDL, which also suppresses enzyme activity (4), is able to compete with LDL for binding, whereas HDL, the lipoprotein-deficient fraction of normal serum, and abetalipoproteinemic serum, which do not suppress enzyme activity (4), do not compete effectively for binding; and (*iii*) cells from homozygotes with FH, which are resistant to LDL-mediated suppression of HMG CoA reductase activity, are also deficient in specific LDL binding.

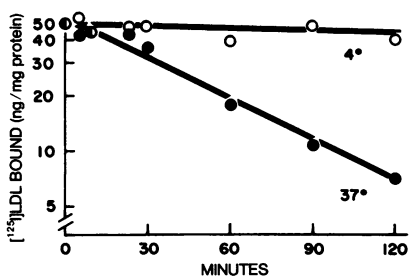

FIG. 7. Semilog plot of the release of [^{125}I]LDL bound to intact normal fibroblasts. On day 6 of cell growth, the medium was removed and the cell monolayers were incubated at 4° in 2 ml of buffer B containing 5 μg/ml of [^{125}I]LDL. After 3 hr, cell monolayers were washed as described in *Materials and Methods* and 2 ml of fresh buffer B containing 5 μg/ml of native LDL was added (0 time). Cells were then incubated at either 4° (○) or 37° (●) and at the indicated time the medium was removed, the cell monolayers were washed once with 2 ml of chilled buffer containing 50 mM Tris·HCl (pH 7.4) and 0.15 M NaCl, and dissolved in 0.1 N NaOH for measurement of the remaining radioactivity as described in *Materials and Methods*.

Although binding of [^{125}I]LDL to normal intact fibroblasts appears to represent an interaction of LDL with a physiological "receptor," the molecular mechanism by which binding leads to suppression of synthesis of HMG CoA reductase molecules is not yet known. Further studies are necessary to determine whether binding of lipoproteins to the cell surface is sufficient in itself to achieve this physiological effect or whether there is an additional requirement for cellular entry of intact LDL or one of its components. The product of the temperature-dependent degradation of bound LDL and its physiological significance also remains to be explored.

The defect in high-affinity LDL binding observed in the cells of homozygotes with FH appears to explain our earlier findings of elevated HMG CoA reductase activity (3, 4) and cholesterol overproduction in these cultured cells (3). Thus, the binding defect may represent the primary genetic lesion in this disorder.

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